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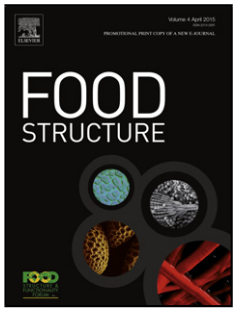

Effects of thermal treatment on collagen present in bovine *M. Semitendinosus* intramuscular connective tissue. Analysis of the chemical, thermal and mechanical properties

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Effects of thermal treatment on collagen present in bovine *M. Semitendinosus* intramuscular connective tissue. Analysis of the chemical, thermal and mechanical properties.

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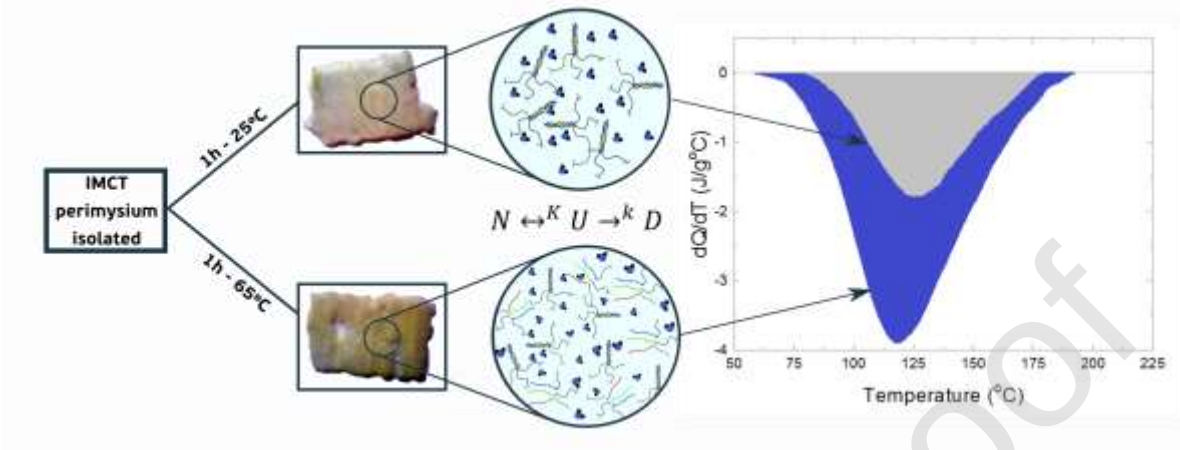
Graphical abstract



Highlights

- The 65°C thermal treatment show as tipping point on collagen of IMCT-perimysium
- Collagen degradation shows that 65°C is critical for significant cooking modification
- Energy to denature remaining collagen increases at the 65°C thermal-treatment

Abstract

There is still interest in understanding how the collagen characteristics of the intramuscular connective tissue (IMCT)-perimysium contributes to the tenderness and tenacity in different meat cuts after the cooking process. In the present work, the analysis of the changes in IMCT after thermal treatments is proposed. The study of the thermal treatment (1h) at different temperatures (25-65°C) was designed to focus on the chemical, mechanical and thermal properties of the collagen present in isolated IMCT-perimysium. Collagen degradation degree (*Deg*), the solubility of collagen and proteoglycan components, thermal properties and mechanical stress were studied on treated-IMCT-perimysium. The results showed that collagen *Deg* increased significantly at 65°C. The soluble proteoglycan fraction did not show changes among treatments. Thermal properties presented notorious and significant changes at 65°C. Enthalpies of denaturation (ΔH) increased and denaturation peak temperatures (T_p) decreased in relation with lower thermal-treatments. Finally, there were not significant differences in mechanical properties and load-stress among treatments. *Deg* at 65°C might indicate chemical changes in total collagen; labile populations were solubilized

while the stable population remained in IMCT-perimysium. The effects of thermal treatment at higher temperatures and the presence of meat enzyme should be investigated in the future.

Keywords: Collagen; Intramuscular Connective Tissue; Meat; Thermal-treatment; DSC; Tensile-test

1. INTRUDUCTION

The recent review by Purslow (2018) remarks the interest of studying the characteristics of the thermally-stable fraction of IMCT collagen since the connective tissue is the main component which influences the contribution to the toughness of cooked meat. Moreover, this question is not only interesting for meat sciences. The contributions of the studies and knowledge on the characteristics and thermal changes of collagen fibres of the IMCT-perimysium (IMCT-peri) are also interesting for other areas, like animal production, general food sciences and biomolecules.

The IMCT, composed by extracellular matrix (ECM), supplies the tissues with mechanical strength and elastic properties. It is mainly constituted by collagen, elastin, proteoglycans (PG) and glycoproteins. The collagen fibres are the main IMCT-molecules, which mainly provide biomechanical strength and their stability is regulated by PGs (Nishimura, 2015).

The current hypothesis suggested by Purslow (2014; 2018) is based on the idea that IMCT matrix contains two populations of collagen molecules: one stable fraction, which is a strong thermo-stable pool, possibly due to the presence of high concentrations of mature cross-links and, another that is relatively weak and thermo-labile (soluble collagen). In raw meat, total collagen is present and contributes to the strength of the IMCT. The Purslow´s (2014, 2018) hypothesis assumes that the more stable population will be more resistant to heat and, likely, it could also be the most stable in terms of resistance to proteolysis. The less stable fraction may be more easily denatured and solubilized upon thermal treatment. Moreover, it could be more labile to proteolysis.

After the cooking process, the strength of the cooked material would be defined by the more stable IMCT-collagen population. Consistent with what has been previously described, the more labile collagen fraction would be solubilized during cooking. Finally, the solubilization degree may be a function of the tissue chemical characteristics, cooking temperature and cooking time.

According to the denaturation model of Lumry and Eyring (1954), whenever the cooking temperature increases, the collagen can be denatured in two stages:

$N \leftrightarrow^{K} U \rightarrow^{k} D$ (eq.1)

N = native, U = unfolded and D = denatured molecules, K=equilibrium constant of the reversible native- unfolded step and k= rate constant for the irreversible unfolded-denatured step. As the temperature increases, the native collagen structures unfold and then it is denatured, leaving separate chains into randomly folded structures. Different authors (Bernal and Stanley, 1987; Gerrard,et al. 1987) have observed that the denaturation of the collagen protein is possible only when denaturation temperature is over ≈ 60°C.

In addition, Sun et al. (2006) investigated the collagen thermal denaturation by high resolution second-harmonic method and the results suggested that intra-individual fibril bonds are broken

more easily than the inter-molecular force. Furthermore, they suggest that the denaturation process responds to a "tiger-tail" pattern, which involves the breakage of the crosslinking (intra-molecular bond). This process occurs easier at the ends of the fibrils where not all collagen molecules are linked due to their organization within a fibril bundle (inter-molecular bonds).

On the other hand and in relationship with the mechanical properties, Hull and Clyne (1996) stated that the stiffness (E_c) of the composite along the direction of the fibres is given by a simple "rule of mixture":

$E_c = V_1E_1 + V_2E_2 + V_mE_m$ (eq.2)

where V_1 and V_2 are the volume fraction occupied by collagen fibres, population 1 (thermal stable collagen fibres) and population 2 (thermal labile collagen fibres) and V_m represents the matrix volume, the fractions E_1, E_2 and E_m are the Young´s modulus of each population, respectively.

In the total collagen of raw tissue, both populations are present. The collagen network is located deep into a hydrated PG matrix, and it defines the stress and the breaking tissue point. However, these networks show changes after the cooking process. The relationship between total collagen content, its thermal properties, chemical cross-linking and its interactions with the PG matrix, would contribute to the strength of the IMCT-peri in muscle (Velazquez and Latorre, 2019).

Thermal treatment induces denaturation and solubilization of compounds to a certain degree. After the heating process, it can be assumed that the stress-strain properties of the components are somewhat altered. Gelation of proteins and some water loss could firm up the matrix, making it a little stiffer and stronger, with a reduced failure strain. According to the results of Lewis and Purslow (1989), some collagen fibres in the perimysium become less extensible possibly by straightening out any waviness in the fibre. However, some changes in the interactions of the water molecules in the collagen triple-helices might also be involved. On the other hand, it is unclear how much of the collagen is in the fully denatured state after heating and if some new arrangements of the helical structure occur.

The volume fraction of the composite occupied by native or denatured collagen fibres depends on the muscle characteristics and the thermal treatment, temperature and time of heating. However, there is some collagen that is completely lost from the composite as heat-soluble collagen (collagen coming out from the tissue and present in the cooking fluid). As observed by Latorre et al. (2018) the denaturation temperature shows linear dependence on the heating rate.

According to previous descriptions, the purpose of the present work is to determine how after thermal process, the IMCT-peri matrix responds to the changes in the chemical, thermal and mechanical properties. Particularly, it is interesting to evaluate how the thermal treatment affects the main matrix components, collagen populations (soluble and insoluble fractions) and PG soluble

fractions, and the thermal and mechanical behaviour of the IMCT-peri. To achieve this, a model system was designed in which IMCT-perimysium was treated after its isolation from meat (free of meat proteases). The bovine *Semitendinosus* IMCT-peri was chosen for the present study because the IMCT-peri disposition allows a reproducible preparation of samples.

2. MATERIAL AND METHODS

2.1 Samples, isolation of IMCT-perimysium and thermal treatment

Bovine *Semitendinosus* (ST) muscles from three (3) *Aberdeen-Angus* (body weight at slaughter ≈375kg; pasture-raised with feedlot termination) were obtained from a commercial slaughterhouse (7 days post-mortem). Each animal muscle was transversally cut following the muscle fibre direction (each slice ≈ 2.5 ± 0.2cm thick).

IMCT-peri from bovine ST muscles was isolated. Small strips were obtained from the perimysium surrounding the muscle fibres bundles. Strips were carefully dissected out from raw meat slices, as described by Latorre, et al. (2018). Maximal care was taken to minimize distortion of the connective tissue. IMCT-peri from each animal (n=3) was treated per triplicate at each thermal point (temperature) and it was used for chemical, mechanical and thermal analysis.

The isolated IMCT-peri samples were placed in distilled water (tissue: H_20; 1:20; m:V; g:ml) and cooked at different temperatures in a water bath (MiLAb, Buenos Aires, Argentina). IMCT-perimysium thermal treatment was carried out during 1 h at 37; 45; 55 and 65°C; control samples (untreated) were kept 1h at room temperature (≈25°C). Each sample was placed individually in 50ml tubes and enough separation between tubes was considered to ensure good water circulation. After thermal process, the samples were immediately cooled in a cool water (≈5°C) bath during 15min.

2.2 IMCT-perimysium Collagen Content

After thermal treatment, the IMCT-peri solid fraction and liquid fraction were separated. The collagen content was measured in both fractions: IMCT-peri residue (remaining collagen) and liquid fraction collagen (degraded-solubilized collagen in water during the thermal treatment). The collagen in the water was designated as the IMCT-peri soluble collagen fraction (V_2), and the collagen in the tissue residue was designated as the IMCT-peri insoluble collagen fraction (V_1). The collagen content was evaluated using the entire insoluble tissue residue fraction and the full volume of the liquid fraction. Each fraction was then separately hydrolyzed in HCl (6N) at 110°C for 16h (m:V; 1:10; g:ml). After hydrolysis, samples were neutralized and the hydroxyproline concentration was determined by spectrophotometry using the colorimetric method of Bergman and

Loxley (1963). Collagen content was calculated by using a correction factor of 7.5. The values were expressed as mg Collagen per gram of IMCT-peri wet base. The thermal damage was estimated by the following formula:

$$Deg\ (T_{1h}) = \frac{C_0 - C_{T1h}}{C_0} \quad \text{(eq.3)}$$

where T_{1h} is the temperature of thermal-treatment during 1h, *Deg* is the degree of thermal damage, C_0 and C_{T1h} are the total (liquid and solid fraction) collagen content and current concentration of un-denatured collagen (solid fraction) (Xu, et al., 2008).

2.3 Total Soluble Carbohydrates

The total carbohydrate content in hydrolysed and neutralized water soluble fraction was determined by the phenol-sulphuric method (Dubois, et al., 1956), using D-Glucose as standard curve. Total glucose (mg Glucose per g of IMCT-peri wet base) was used as PG equivalent content (V_m).

2.4 Differential Scanning Calorimetry (DSC)

The thermal denaturation kinetics of the IMCT-peri residue samples were analyzed using a DSC Setaram Evo 131. IMCT-peri residue samples (10-15 mg strips) of hydrated perimysial connective tissue were dried at 37°C for 24h. According to Latorre, et al. (2019) it is possible to study the thermal stability of the wet tissue. However, this drying procedure was followed in order to have an accurate measure of the amount of dry matter of the protein analyzed and to obtain a concentrated sample, producing a stronger DSC signal. This process allowed the detection of small differences among different treatments. It is known that the complete drying of collagen increases the temperature of denaturation to temperatures above 110°C (Finch and Ledward, 1972). Dried perimysial samples were encapsulated in a small sample pan (part n° L7168). Non-isothermal DSC curves were obtained using heating rates of 10°C min^{-1}, in the range between 25 and 300°C using Argon as sweeping gas. An empty pan was used as reference. DSC scans were performed in triplicate and the temperatures are reported as averages. After baseline correction using a polynomial function, enthalpies of denaturation (ΔH) and denaturation peak temperatures (T_p) were determined from the curves.

2.5 Tensile test of residue perimysial connective tissue

For this study, the methodological procedure described by Lewis and Purslow (1989) was followed. The residue IMCT-peri strips were cut with their long axes transversally to the muscle fibre axes and they were carefully trimmed with a scalpel obtaining a dumbbell- shaped strip with a narrower

central region approximately 6mm long and 2mm wide. This dumbbell-shaped strip ensures that tensile failure occurs in the middle of the specimen and not at the gripping points. The wider ends of the specimens were attached to aluminium templates with cyanoacrylate adhesive glue to permit handling and transfer into the mechanical testing device without undue distortion. The template was then glued, using cyanoacrylate glue, into two clamps, one attached to a motor and the other to the force transducer of the testing device. Prior to testing, the length and width of each sample was carefully measured using Vernier calipers. The sides of the template were cut to leave only the perimysial strip bridging the clamps and the specimens immediately extended to fracture at a constant rate of 75mm/s. Loads and extensions were monitored using a high-resolution A/D converter (ADC-20, Pico Technology, Cambridge, UK) running under PicoLog data acquisition software. The maximum load observed was recorded at the breaking load and the extension at maximum load was taken as the breaking extension. At the end of the mechanical test, the free length of each sample was cut from the clamps and dried at 37°C for 24h to obtain the dry weight. The amount of collagen in this dried sample was determined using the procedure outlined in Section 2.2.

2.6. Statistical analysis

All experiments were performed at least in triplicate unless otherwise specified. The results are reported as the average, standard deviation and standards errors (SE). Comparisons among the results from each heating temperature were performed by one-way ANOVA with Post-hoc Dunnett's post-test (α 0.05). Statistical analysis was carried out using Graph-Pad Prism version 5.00 for Windows, Graph-Pad Software, San Diego, California USA http://www.graphpad.com .

3. RESULTS AND DISCUSSION

3.1. Collagen degradation degree (*Deg*)

Significant differences in the collagen *Deg* (eq. 3) were detected at 65°C thermal-treatment (Table 1). However, the IMCT-peri collagen degradation and water solubilization had a small, unaccountable increase at 45°C (non-significant). Similar collagen solubility (%) behaviours were observed at 40°C (Chang, et al., 2011a) and 55°C (Li et al., 2010) in beef ST muscle.

It is known that the complete collagen can be denatured at ≈60-65°C (Bernal and Stanley, 1987; Gerrard et al., 1987). Privalov (1982) mentioned that both native and denatured proteins are the macroscopic states which are dependent on different external variables like pH, temperature and ionic strength. Collagen can also be dissolved at the body temperature and with the same salt level

as in a living body (Leikina et al., 2002). Moreover, the authors suggested that collagen helices could begin to unfold once secreted from cells and initial microunfolding of least stable domains, would trigger the self-assembly of fibres where the helices are protected from complete unfolding. Finally, Leikina et al. (2002) proposed that helices of collagen fibres in the cells could melt and refold locally when needed. That is, the collagen molecules could separate at a temperature lower than body temperature and aggregate in the proper sequence in a living body. Bovine body temperature is 38.5°C. Therefore, it would be possible that the treatment at 37°C for 1h induced collagen denaturation. According to Leikina et al. (2002) this population is likely monomeric collagen, newly synthesized and just incorporated into fibrils. Furthermore, it is unstable and it can be denatured if exposed at 37°C for enough time (2 to 3 days).

In addition, Chang, et al. (2011b) in beef ST muscle heated from 40 to 90°C observed an increase in collagen solubility with an increase in the heating temperature, particularly when it was higher than 65°C. They also suggested that a heating temperature of 60°C and 65°C is critical for altering the characteristic of collagen. Moreover, Christensen et al. (2013) observed that in ST the percentage of heat-soluble collagen increased with increasing the temperature from 53°C to 63°C in cows.

The present results are according to the current knowledge which indicates that a fraction of IMCT-peri collagen is heat-solubilized. However, it is usually only a small fraction, being the remaining, insoluble fraction, the predominant. Furthermore, IMCT collagen thermal denaturation temperature is usually in the range of 60-68°C. Nevertheless, collagen can achieve denaturation at lower temperatures (between 60-65°C).

On the other hand, this significant increase observed at 65°C could be responding to the tiger-tail collagen denaturation model of Sun et al. (2006). The authors suggest that the bonds within the individual fibrils are broken easier than the inter molecule forces joined fibrils. The collagen denaturation process involves the breaking of the cross-linking force holding the collagen molecules into fibril, and individual N↔U→D steps. They also indicated that it could be possibly to think that this occurs more easily at the ends of the fibril where not all collagen molecules are linked due to their staggered arrangement within a fibril bundle. Even Purslow (2018) has suggested that the collagen denaturation is a multistep, non-equilibrium process that is highly dependent on the heating rate and can occur at 55-60°C in slow heating regimes.

3.2 Matrix compound fractions

Considering the insoluble collagen, soluble collagen and soluble PG as main matrix compounds, the fractions (volume) of each of them were evaluated as per total compound (Σ mg (Soluble Collagen + Insoluble Collagen + Soluble Glucose / g IMCT-peri wet base (w.b.)). Figure 1 shows the

percentage volume of each fraction at different temperatures. The results showed that only at 65°C there are significant differences with respect to the control (25°C) for soluble ($P<0.0001$) and insoluble ($P=0.0074$) collagen percentages. However, for soluble PG significant differences were not detected among any treatment ($p=0.089$).

The results by Lewis et al. (2010) with corneal tissue suggest that the PG chain can often extend between the collagen fibrils, confirming the presence of contact points between collagen fibres and PG. The authors also suggested that the PG occupies the extra-fibrillar space and could be acting to tether the collagen fibrils giving them spatial order. Nevertheless, the authors do not include thermal treatment. Finally, the present results on PG are consistent with the suggestion that the PG ($S_m V_m$) is negligible and does not contribute to matrix load stress. The IMCT-peri thermal treatment results, V_m (PG), V_2 (soluble collagen) and V_1 (insoluble collagen) fractions are novel. Nevertheless, further PG studies are necessary in beef IMCT-peri.

3.3 DSC test: Thermal characteristics of IMCT perimysial collagen

After thermal-treatment, all samples showed that the collagen remaining still has a native, undenatured state and this can be observed in the corresponding DSC curves (Figure 2). Table 2 shows the average values for the total denaturation energy (ΔH) per g of the remaining IMCT-peri collagen, and the peak temperatures (T_p) of the denaturation endothermic reaction for each thermal-treatment (TT) temperature. The total denaturation energy per gram of IMCT-peri (Figure 3) as well as per gram of remaining collagen showed a significant increase after thermal-treatment at 65°C. The T_p pattern of variance showed that in the uncooked material (25°C) and in the material treated at 65°C, both T_p were lower than that of the other treatment temperatures and similar between them (124°C and 118°C, respectively). However, in 1h thermal-treatments at 37, 45 and 55°C, the peak temperature for denaturation of the remaining collagen showed an increase (≈138°C). According to these results, the thermal behaviour of the ST IMCT-peri looks one way for TT at 25°C, other different and equally between them for the samples subjected to TT at 37, 45 and 55°C, and finally shows different properties for TT at 65°C .These results could indicate that there are at least two phases of denaturation; one close to the body temperature of the animal (37°C) and another at ≥ 60-65°C. This could explain why the curves for the 37, 45 and 55°C TT samples do not show significant differences from each other. Only when the next temperature denaturation threshold is reached (> 60-65°C) the next change can be observed. Eventually, the T_p could be the manifestation of the hysteresis characteristic for all cooperative equilibrium. However, in the present work, the characteristics of Type I chains were not analyzed by SDS-page. On the other hand, the T_p differences after thermal-treatment might be accounted by the changes produced in the structural

organization of the collagen (Bernal and Stanley, 1987). Privalov (1982) has indicated that water molecules participate in maintaining the collagen helix. In addition, according to Kopp and Bonnet (1986) when the collagen is thermally treated, intramolecular hydrogen- bonds are cleaved, inducing shrinkage of the collagen fibres followed by solubilisation and gelatinization. More recently, Chang et al. (2011b) studied DSC thermal melting in ST perimysium and endomysium in cooked meat at different temperatures both in water bath and microwave. The authors suggested that the decrease in the perimysium collagen thermal melting values could be explained by a weakening of the average stability of collagen, by gelation and denaturation heating effect.

The thermal-treatment at 65°C not only presented a T_p decrease in relationship with other thermal-treatments, but also showed a significant increase in ΔH. Similar changes were observed by Latorre et al. (2019) in ST-IMCT-peri after different cooking times at 60°C. The current DSC experiments were carried out on dehydrated specimens to reduce the possibility that gross changes in hydration of the collagen would influence the results. However, small amounts of water bonds (water mono-layer) could be present into the collagen molecules. Several researchers (Bergman and Loxley, 1970; Kopp, and Renou, 1990; Rochdi et al., 1999) have indicated that in the collagen water content variation produced marked changes in ΔH and T_p, showing that as the moisture content increases, ΔH increases and T_p decreases. Kopp et al. (1990) observed that at higher unfrozen water content on collagen-IMCT, the T_p decreases and the ΔH increases. The authors suggested that these changes could respond to differences in hydrogen bonds and/or hydrophobic bonds.

On the other hand, the collagen dynamic model of Streeter and de Leeuw (2011), suggests that the collagen fibril is based on two important types of hydrogen bond interactions. This model proposes inter-protein hydrogen bonds (between neighbour tropocollagen molecules) and bridging water molecules linking two adjacent tropocollagen molecules by forming hydrogen bonds to each of them simultaneously. This could suggest that in control samples (25°C) there are optimal hydrophilic and hydrophobic interactions.

It is known that while the samples are heated, the collagen starts to destabilize. However, the thermal treatment may not be enough to denature collagen (there are still higher hydrophobic interactions, high T_p). For the lowest TT temperatures used in this work (37ºC, 45ºC and 55ºC), the thermal treatment may indicate that treating the samples at these temperatures for 1h was not enough to denature the thermal soluble collagen population. Nevertheless, for the TT 1h at 65ºC, a temperature close to the collagen denaturation temperature (60-65 °C), the denaturation of the soluble collagen population can be possible. This change could be attributed to some of the following alterations: an increase in random water-protein interaction caused by disruption of the fibrillar collagen arrangement, collagen-partial denaturation (gelatine-protein), and /or re-folding of

mix collagen helix after cooling. Finally, these changes might be a response to different DSC pattern for TT-65°C.

The results reported here might suggest that the thermal treatment 1h at 65°C produces irreversible changes on the collagen proteins (denaturation). Previous works, observed similar collagen Tp changes with collagen water content and/or hydrogen-bond interactions (Kopp and Renou, 1990; Rochdi et al., 1999).

The present results could indicate that the 'remaining collagen structure' after each cooking treatment and following the cooling step, might have significant differences in the number of hydrophobic and hydrophilic (hydrogen bonds) interactions and/or differences in the mix of native and denatured triple helices. To confirm this, further studies of the water molecule content and/or water-collagen interactions and SDS gel electrophoresis assays are necessary.

3.4 Tensile properties of isolated perimysium

Table 3 shows the load required to break individual sheets of perimysium as a function of heating temperatures during 1h. The load results are expressed as maximum load (L_{Max}) and as load required for reaching 50% extension ($L_{50\%}$), expressed as g force by mg of collagen in the tensile sample. Furthermore, Table 3 shows the total extension percentage at each point, for the $L_{50\%}$ (g/ mg Collagen) strength values were not detected differences between thermal-treatments ($p>0.05$). However, the behaviour observed might indicate a slight rise from the raw state in the first hour of cooking over 65°C. Latorre et al. (2019) have observed similar increase at the first hour at 60°C. The current results, like those of previous studies, reveal the existence of great differences among animal samples and, as a consequence, the size of the standard errors in these measurements precludes any statistical significance from being reached.

Chang et al. (2011 a) observed by SEM micrograph that when meat samples were heated to 40ºC there were not significant changes in the structure of the IMCT compared to the unheated samples. However after heating to 50ºC, granulation changes of the perimysium began. Finally, they observed that for temperature treatments up to 60ºC, the perimysium was less discernible. This microstructure changes could be occurring in the IMCT-peri and affecting the maximum load. Although not significant differences were observed, treatment at 55°C showed the lower load and lower extension in relationship with the other thermal treatments.

3.5 IMCT-peri Collagen Degradation Degree *vs* Thermal and Mechanical properties

Fig. 3 shows the Collagen *Deg* evaluated in relationship to collagen thermal parameter (ΔH J/ g IMCT-peri dry base (d.b.)) and the tensile-test property (Load at 50%; g/g IMCT-peri d.b.) post-

thermal treatment at each temperature (Fig 3-a and -b, respectively.) The ΔH and $L_{50\%}$ results were previously discussed. The results on DSC showed significant differences at higher temperatures. However, according to the mechanical test significant changes did not occur. In addition, the correlation between Collagen *Deg* values showed interesting results suggesting that the thermal-treatment not only produces collagen degradation (population-2 solubilization) but also structural changes in IMCT-peri collagen fibrils. The collagen denaturation process involves the breaking of the cross-linking force holding the collagen molecules into fibril, and individual N↔U→D steps. According to Sun et al. (2006) it is possibly that this occurs more easily at the ends of the fibril where not all collagen molecules are linked due to their staggered arrangement within a fibril bundle. According to all previously exposed, we could suggest that the cooking process produces IMCT-peri collagen thermal denaturation and solubilization of population 2 of collagen. Eventually, for the collagen population 1, it is possible that changes in the chemical interactions of collagen at <65°C might be taking place. As a consequence, different numbers of hydrogen bonds (hydrophilic interactions) could be present too. The *Deg* and stress (tensile-test) correlation results could indicate that these chemicals and possible molecular structural changes, do not show a significant impact on IMCT-peri tensile mechanical behaviour.

4. CONCLUSION

According to the results from this study the thermal treatment at 65°C produced significant changes of the main IMCT-peri components. There was an increase in the connective tissue collagen degradation degree. The soluble PG fraction was similar among treatments. PG solubilized at room temperature and variations among the treated samples were not observed. Although, it is known that the PGs are involved in the stability of the collagen fibre network, the results for room temperature (25°C)-solubilized PG could suggest that the V_m is relatively low, and the matrix is unlikely to transfer enough load to the fibres to cause them to break. The thermal denaturation collagen properties were modified by the one hour thermal treatment. The structural collagen molecule changes were studied by ΔH and T_p parameters obtained from DSC tests. Interesting results were observed in thermal behaviour of the ST IMCT-peri looks one way for TT at 25°C, quite differently for samples with TT at 37, 45 and 55°C, and finally show differently for TT at 65°C. These results could indicate that there are at least two phases of denaturation; one near to the body temperature of the animal (37°C) and the next change to would be to ≥ 60-65°C.

Thermal treatment at 65°C showed a significant ΔH increase and T_p decrease, changes of these parameters are in agreement with the literature. These changes could suggest that above 65°C, the remaining collagen may represent the thermal-stable collagen population and may indicate different

amounts of hydrophobic and hydrophilic (hydrogen bonds) interactions. However, further studies are necessary to evaluate chemical interactions.

The IMCT-peri collagen tensile results did not show differences among treatments. However, it is known that variations in the quantity and thermal stability of collagen in IMCT play a role in the variations observed in cooked meat tenderness. Finally, the IMCT-peri results suggest that thermal treatment at 65°C has major effects on collagen, chemical-changes and thermal denaturation parameters. This might indicate that heating temperature of 65°C was critical for affecting the chemical and thermal collagen properties of bovine ST IMCT-peri. These results are according to others studies made in beef ST muscle. Nonetheless, the effects of thermal treatment at temperatures higher than 65°C as well as in the presence of others meat components (myofibril, sarcoplasm proteins) should be further investigated using this isolated IMCT-peri system.

"Declarations of interest, none"

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Figure 1-Fraction of thermal soluble proteoglycan (PG); soluble collagen and insoluble collagen; matrix volume (V_m), thermal stable collagen fibres population (V_1) and thermal labile collagen fibres population (V_2)

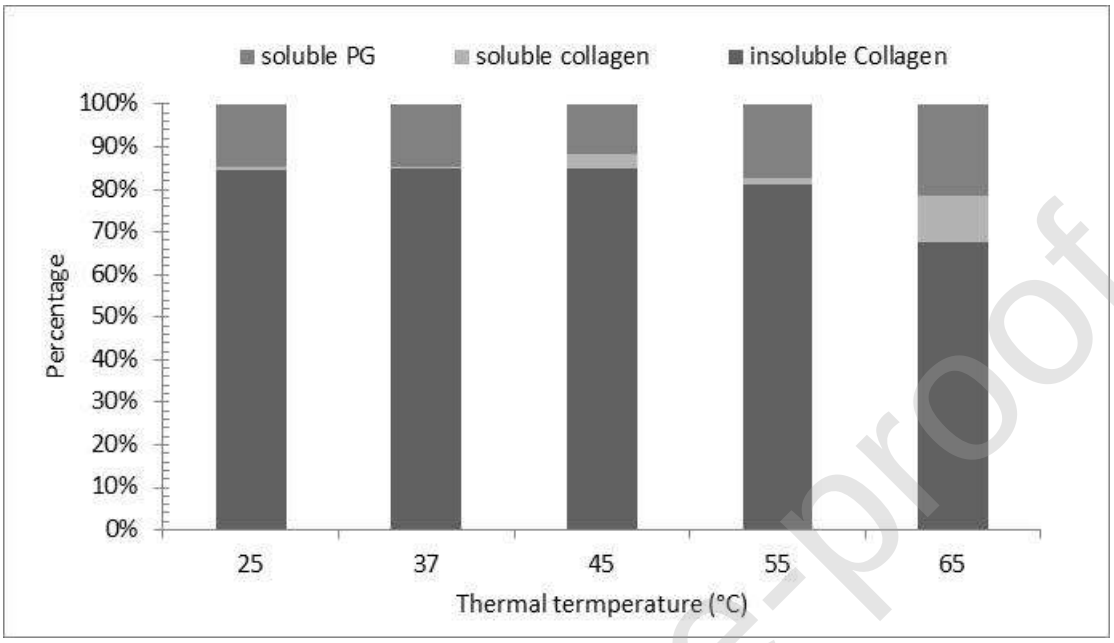

Figure 2. Normalized DSC curves recorded on heating at 10 °C min^{-1} after 1h thermal treatment at different temperatures. Parameters calculated from the curves are shown in Table 2.

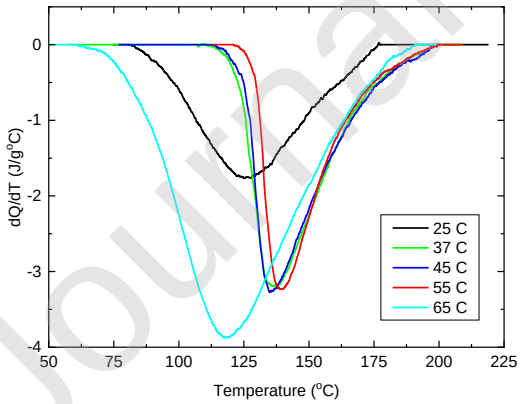

Figure 3 Collagen degradation degree (*Deg*) correlations with **a-**thermal properties (enthalpies of denaturation, ΔH) and **b-**mechanical properties ($L_{50\%}$ Load required for reaching 50% extension) in IMCT-peri after 1h thermal treatment at different temperatures (J/g IMCT-peri dry base and g/g IMCT-peri dry base; respectively).
The errors bars represent ± one standard deviation (±sd); R^2: square correlation coefficient.

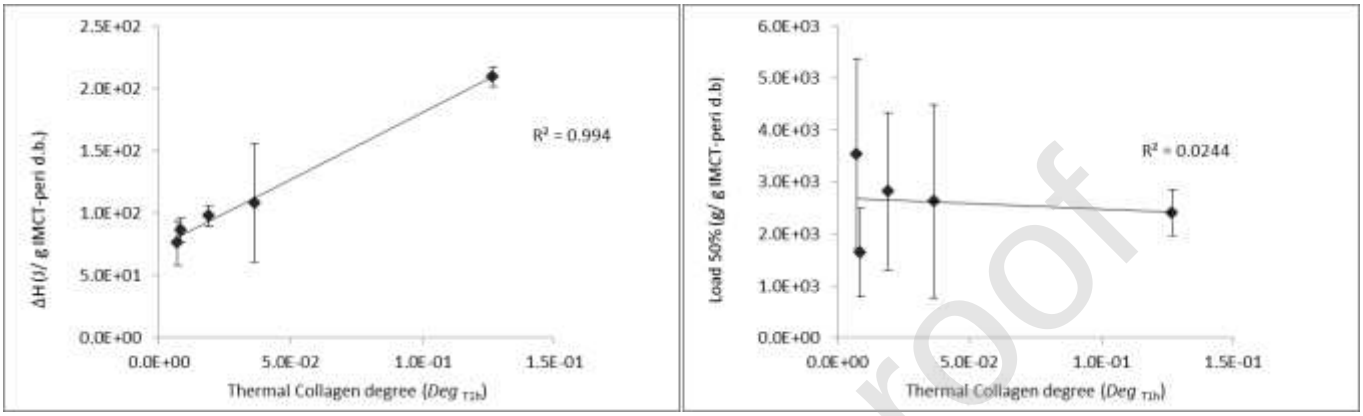

Table 1 Thermal Collagen degradation degree.

°C	*Deg** $_{(T1h)}$
25	0.01±0.001[a]
37	0.01±0.001[a]
45	0.04±0.010[a]
55	0.02±0.020[a]
65	0.14±0.050[b]

All fractions were quantified by triplicate (n=3). Tabulated values are average and ± standard deviation. Different superscript letters indicate significant differences ($p < 0.05$) among samples (One-way ANOVA-test with Post-hoc Dunnett´s post-test).
**Deg*: degree of thermal damage. *Deg* $_{(T1h)}$ $=(C_0-C_{T1h})/ C_0$. C_0 and C_{T1h} are the total (liquid and solid fraction) collagen content and current concentration of un-denatured collagen (solid fraction). T_{1h} is the temperature of thermal-treatment during 1h.

Table 2 Thermal IMCT-perimysium properties after different temperature treatments during 1h. ΔH (J/g collagen) = total denaturation energy expressed as J per g collagen in the dry IMCT-perimysium (mean ± standard error, n=3 for all points). T_p is the peak temperature (°C) of the endothermic reaction in DSC curves.

°C	ΔH J/g Collagen	T_P °C
25	379 ± 264 (152)[a]	124.0 ± 4.4 (2.5)[a]
37	487 ± 52 (30)[a]	139.7 ± 4.0 (2.3)[b]
45	527 ± 252 (146)[a]	138.0 ± 2.6 (1.5)[b]
55	527 ± 312 (180)[a]	135.7 ± 1.2 (0.7)[b]
65	2816 ± 1219(704)[b]	117.7 ± 1.5 (0.9)[a]

All temperature treatments were quantified by triplicate (n=3). Tabulated values are average ± standard deviation, with standard error of the mean (SE) in brackets. Different superscript letters indicate significant differences ($p < 0.05$) among samples (One-way ANOVA-test with Post-hoc Dunnett´s post-test). ΔH: denaturation energy (J/g Collagen); T_p: peak temperature.

Table 3- Tensile test parameters of IMCT-perimysium post temperature treatment during 1h.

°C	Extension (%)	L_{Max}/m g/mg Collagen	$L_{50\%}$/m g/mg Collagen
25	80 ± 10 (6)	86.7 ± 77.9 (45)	13.8 ± 1.7 (1.0)
37	99 ± 26 (15)	95.3 ± 40.8 (25)	9.7± 5.8 (3.3)
45	102 ± 17 (10)	137.7 ± 27.1 (15.6)	12.3 ± 7.8 (4.5)
55	81 ± 15 (9)	48 ± 30.3 (17.5)	12.8 ± 3.0 (1.7)
65	113 ± 3 (2)	134.5 ± 55.7 (32.2)	32.1 ± 14.3 (8.3)

All temperature treatments were quantified by triplicate (n= 3).Moreover each animal was quantified by quintuplicate (n= 5 per animal) at each thermal treatment. Tabulated values are average ± standard deviation, with standard error of the mean (SE) in brackets.
* Load (g) per milligram of collagen. L_{Max} Max Load at breaking point. $L_{50\%}$ Load required for reaching 50% extension.